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The Effects of Holothurin, a Steroid Saponin of Animal Origin, on Krebs-2 Ascites Tumors in Swiss Mice

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Introduction

THE preparation and general characteristics of the water soluble, thermostable factor, Holothurin, from the Cuvierian organ of the sea-cucumber, *Actinopyga agassizi* Selenka, have been described by Nigrelli (1952)[2] and by Nigrelli, Chanley, Kohn & Sobokta (1955). Nigrelli & Zahl (1952) reported that Holothurin inhibited growth of certain Protozoa and that *in vitro* treatment of Sarcoma 180 cells with this substance markedly reduced the subsequent growth of these tumor cells after inoculation into Swiss mice. The present preliminary report is primarily concerned with the *in vitro* and *in vivo* effects of Holothurin on the Krebs-2 ascites tumor in Swiss mice.

Materials and Methods

The Krebs-2 ascites tumor, carried in Swiss mice and in a breeding colony of Highline, ICR Swiss mice, was obtained in 1952 from Dr. T. S. Hauschka. The tumor has been maintained by weekly intraperitoneal inoculations of 0.1-0.2 ml. of ascitic fluid into 10-12 mice. All animals in these experiments were obtained from the original breeding mice by cousin matings, and weighed 18-25 grams at the beginning of the experiments.

The method recommended by Goldberg, Klein & Klein (1950) for counting the tumor cells and for the inoculation procedure was used. The donors of ascitic fluid had carried the tumor for at least 7 days but not more than 13 days. A uniform inoculum of 2×10^6 tumor cells suspended in sterile saline, as suggested by Sugiura (1953), was injected in all experimental animals. No remission has occurred in more than 2,000 mice inoculated with 1 to 44 $\times 10^6$ tumor cells.

Holothurin was dissolved in physiological saline and autoclaved for 30 minutes at 20 lbs. pressure. The method of Nigrelli & Zahl (1952) was used for treating the tumor cells *in vitro;* varying amounts of Holothurin were added to tubes containing 20×10^6 tumor cells diluted with sterile saline. The tubes were incubated at 99°F and shaken for 1 hour. Control tubes were similarly treated.

In the *in vivo* experiments 0.01, 0.05, 0.1 and 0.2 mg. of Holothurin were injected daily or spaced over 7-21 days. Repeated injections were made intraperitoneally on alternate sides of the abdomen. Controls with and without tumor cells were maintained simultaneously. Fifty-six mice were also inoculated with tumor cells in the subcutaneous areas of the axillary and inguinal regions. These did not survive for more than two and one-half months.

Results

Toxicity of Holothurin.—Intraperitoneal injection of 0.2 mg. of Holothurin in sterile saline was lethal in 48 hrs. for 6 female mice; injection of 0.1 mg. into 12 female mice caused no deaths. The safe upper limit for injection of Holothurin was taken as 0.1 mg.

In Vitro Experiments.—No deaths were observed in 60 days in female mice inoculated with 2×10^6 tumor cells treated with 0.1 mg. of Holothurin. These mice produced normal litters when mated; the offspring, however,

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TABLE 1. STANDARDIZATION OF MEAN SURVIVAL TIMES AND BODY WEIGHT GAINS OF SWISS MICE CARRYING THE KREBS-2 ASCITES TUMOR*

No. of Mice	Sex	No. Mice/Cage	No. of Cages	Range of Mean Survival Time for 31 Cages	Mean of 21 Mean Survival Times
186	F	6	31	9.3-15.1 Days	11.9 Days

Range of Days of Death of 186 Mice	Mean Wt. of 186 Mice on Day of Inoculation	Range of Single Wts. of 186 Mice on Day of Inoculation	Range of 31 Mean Wts. of 31 Cages of Mice on 10th Day after Inoculation	Mean of 31 Mean Wts. on 10th Day after Inoculation
6-26	22.0 gm.	18-25 gm.	26-36 gm.	32 gm.

*All mice were inoculated with 2×10^5 tumor cells diluted with sterile saline. Saline controls not included.

TABLE 2. EFFECTS OF HOLOTHURIN ON SWISS MICE CARRYING AN INOCULUM OF 2×10^6 KREBS-2 TUMOR CELLS

No. of Mice	Sex	Mg. Holothurin Injected	No. of Holothurin Injections	Mean Survival Time (Days)*	Mean Wt. on 1st Day of Tumor Cell Inoculation	Mean Wt. on 10th Day after Inoculation
12	F	Controls	Controls	M 13.7 R 10-19	M 21.7 gm. R 18-25	M 30.2 gm. R 26-33
6	F	0.01 mg.	7 injections in 8 days	M 8.5 R 5-10	M 21.6 R 19-23	
6	F	0.05 mg.	7 injections in 8 days	M 19.5 R 14-28	M 20.0 R 18.5-21	M 23.2 R 19.3-25.5
6	F	0.10 mg.	7 injections in 16 days	M 23.5 R 5-35	M 22.7 R 19-25	M 20.6 R 16.7-25.2
6	F	0.10 mg.	10 injections in 21 days	M 34.6 R 8-51	M 21.9 R 19-25	M 20.6 R 18-22.5

*M is the mean day of death or mean weight of mice; R is the range of variation

TABLE 3. EFFECTS OF HOLOTHURIN ON SWISS MICE CARRYING AN INOCULUM OF 2×10^6 KREBS-2 ASCITES TUMOR CELLS

No. of Mice	Sex	Mg. Holothurin Injected	No. of Holothurin Injections	Mean Survival Time (Days)	Mean Wt. on 1st Day of Tumor Cell Inoculation	Mean Wt. on 10th Day after Inoculation
6	F	Controls	Controls	M 11.1 R 7-15	M 23.3 gm. R 21-24.5	M 29 gms. (7 days) R 23-33
24	F	0.1 mg.	10 injections in 20 days	M* 25.4 R 12-45	M 23.3 R 20-25	M 23.5 R 20.5-27.5

*The mean survival time is for 20 mice since 4 of the treated mice have lived for more than 6 months with no gross indication of a tumor.

showed no resistance to the tumor. In a control group of 6 mice, all were dead by the 18th day, with a mean survival time of 12.5 days.

Two deaths occurred in a group of 12 female mice that were given 2×10^6 tumor cells treated with 0.05 mg. of Holothurin. The amount of material needed to irreversibly inactivate 20×10^6 tumor cells at 99°F. for 1 hr. is between 0.05 and 0.1 mg.

In Vivo Experiments.—The efficacy of Holothurin as a tumor growth inhibitor was judged on the basis of a comparison of the body weight gain on the 10th day after tumor cell inoculation and of the mean survival time in treated and untreated mice.

A group of 186 female mice (31 cages of 6 mice/cage) was inoculated with the standard amount of tumor cells. All mice died between the 6th and 26th day after inoculation. The results, shown in Table 1, were used as the baseline for comparison with the individual controls and experimental mice. Another group of 120 animals gave similar results. Two mice in this group, however, survived past the 24th day, dying on the 33rd and 36th day after inoculation.

A series of 7 injections of 0.01 mg. of Holothurin started on the day after inoculation and continued for 7 days gave an apparent reduction of the mean survival time (Table 2). However, this mean survival time (8.5 days) falls very close to the lower limit of variation found among the 186 control mice (see Table 1). It is impossible, therefore, to conclude that there has been a true reduction in mean survival time with the amount of Holothurin used in this series.

A similar series of injections of 0.05 mg. of Holothurin caused a slight increase in mean survival time and a marked reduction in gains in body weight on the 10th day after inoculation (Table 2).

The daily injection of 0.1 mg. Holothurin was found to be lethal. However, a series of 7 injections given on alternate days resulted in some increase in mean survival time and a marked reduction of mean body weight on the 10th day. A series of 10 injections of 0.1 mg. spaced over 21 days produced a considerable increase in mean survival time and a very marked reduction of mean body weight gain on the 10th day after inoculation (Table 2).

The results from another group of 24 female mice inoculated with the standard amount of tumor cells and given a series of 10 injections of 0.1 mg. of Holothurin over a period of 20 days are shown in Table 3. The increase in survival time and the marked reduction in gain of mean body weight on the 10th day is clearly evident when compared to the experimental and

baseline controls. Four of the 24 treated mice showed no observable growth during a period lasting over 6 months.

Discussion

The unequivocal validity of a test based on 186 animals is, of course, open to question. The repetition of the test on 120 animals gave nearly identical values, but there were two control animals that survived past the 24th day after inoculation, dying on the 33rd and 36th day. There have been no remissions, however, in such inoculated animals, nor in more than 2,000 mice inoculated with Krebs-2 ascites tumor cells in amounts from 1 to 44 $\times$ 10^6.

The four remissions observed cannot be entirely explained by accidental subcutaneous inoculations. Of a group of 56 mice inoculated subcutaneously in the axillary and inguinal regions, none were living after two and one-half months.

Summary

Injection of 0.2 mg. of Holothurin in sterile saline into female Swiss mice (Highline, ICR) is lethal within 48 hrs.

The treatment of 20 $\times$ 10^6 Krebs-2 ascites tumor cells *in vitro* with 0.1 mg. of Holothurin by shaking such suspensions for 1 hr. at 99°F. results in inactivation of the tumor cells. The inoculation of such treated cells into normal mice produced no observable growth during a period of 60 days.

The daily injection of 0.01 mg. of Holothurin for 7 days, beginning the day after inoculation of the tumor cells, had no effect on the progressive growth of the tumor.

The daily injection of 0.05 mg. of Holothurin for 7 days, beginning the day after inoculation of tumor cells, brings about an increase in survival time and a marked reduction of mean body weight gain on the 10th day.

The spacing of one day between injections of 0.1 mg. of Holothurin in a series of 7-10 injections results in a marked increase in mean survival time and reduction in mean body weight gain on the 10th day after inoculation.

Four remissions of the tumor of more than 6 months duration occurred in a group of 24 mice treated with 0.1 mg. of Holothurin in a series of 10 injections over a period of 20 days.

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